# METHOD FOR CLONING PCR PRODUCTS WITHOUT RESTRICTION OR LIGATION ENZYMES

## CROSS-REFERENCE TO RELATED APPLICATION

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## GOVERNMENT RIGHTS

This invention was made with government support awarded by the National Science Foundation (Grant No. MCB-0133258). The government has certain rights in the invention.

## FIELD OF THE INVENTION

The present invention relates to methods for cloning polymerase chain reaction products using site-specific recombination.

## BACKGROUND OF THE INVENTION

The use of the polymerase chain reaction (PCR) in nucleic acid research has provided a convenient way to amplify and construct recombinant DNA. PCR is an *in vitro* method of nucleic acid synthesis by which a particular segment of DNA can be specifically replicated. The method involves two oligonucleotide primers that flank the DNA fragment to be amplified and repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers with DNA polymerase. These primers hybridize to opposite strands of the target sequence, the primers being oriented so that DNA synthesis by the polymerase proceeds across the region between the primers. Since the extension products themselves are also

complementary to and capable of binding primers, successive cycles of amplification essentially double the amount of DNA synthesized in the previous cycle. The result is an exponential accumulation of the target fragment, approximately $2^n$, where n is the number of cycles of amplification performed.

For most applications, PCR products need to be cloned into a vector. Cloning of PCR products into a vector generally involves the incorporation of restriction sites at the ends of PCR products or blunt-ended ligation of PCR products into the vector.

Several technical problems exist with the use of such techniques for cloning PCR products. Restriction sites may exist within the DNA to be cloned and provide targets for cleaving the DNA to be inserted. Restriction sites at the ends of PCR products are often inefficiently cleaved by restriction enzymes because they are too close to the end of the DNA fragment. The PCR products lack part of the binding region for the restriction enzyme to contact the DNA template. The introduction of extraneous nucleotides at the 3' end of the amplified DNA fragments by *Taq* DNA polymerase leads to relatively low overall efficiency of the blunt-end ligation reaction. The incorporation of extraneous nucleotides would alter a reading frame and constructs a copy that includes the undesired nucleotide. The efficiency of blunt ended ligation is poor for the following reasons: i) the $K_m$ for the activity of T4 ligase on blunt-ended DNA is nearly 100 times higher than its $K_m$ on DNA with cohesive ends, thus, ligation of blunt-ended DNA requires a high concentration of enzyme and a high concentration of DNA ends (greater than 1 microgram), therefore very large amounts of the fragment to be cloned are needed, ii) during blunt-end ligation, a fraction of the plasmid vector will recircularize and contribute to non-recombinant backgrounds, and iii) because of the high concentration of the fragments to be cloned, many recombinant plasmids will contain more than one insert of foreign DNA. Furthermore, blunt ended cloning is directionless. In many applications, the orientation of the DNA fragment in the PCR construct is crucial for gene expression.

Thus, cloning of PCR products is often not straightforward. Moreover, it generally requires several steps, including DNA fragment purification, an overnight ligase-dependent ligation and colony selection to determine the correct orientation of the insert. These steps are usually labor intensive, time consuming and/or of low efficiency. It will usually take at least one day to prepare DNA fragments and another day for the ligase reaction.

There is a need for more efficient methods for cloning PCR products that do not require the use of restriction enzymes, ligation enzymes, or DNA purification steps.

## SUMMARY OF THE INVENTION

In one aspect the invention provides methods for cloning polymerase chain reaction (PCR) products into a target sequence that do not require the use of restriction or ligation enzymes *in vitro*, or DNA purification steps. According to the invention, the PCR products may be cloned directly from the PCR reaction. According to these methods, a PCR product is cloned into a target sequence *in vivo* using a site-specific recombination system. In some embodiments, the methods include the steps of (1) providing a PCR product flanked by a first site-specific recombination site and a second site-specific recombination site; and (2) transferring the PCR product into a cell comprising a target sequence flanked by a first recombination site partner and a second recombination site partner, and at least one recombination protein that mediates recombination between the first site-specific recombination site and the first recombination site partner, and between the second site-specific recombination site and the second recombination site partner.

The PCR products are typically generated using a set of primers to provide the first and second recombination sites to the PCR product. In some embodiments, the PCR products are linear. The target sequence may be a genomic sequence or a plasmid sequence. The target sequence may be transferred into the cell simultaneously with the PCR product.

In some embodiments, the site-specific recombination system is the integrase/attachment system from bacteriophage lambda. In some embodiments, the cell is an *E. coli* bacterial cell. The PCR products may be transferred into a cell that further comprises at least one other site-specific recombination protein, such as Integration Host Factor.

In a further aspect, the invention provides site-specific recombination kits including vectors, primers, and cells for cloning PCR products *in vivo*.

## BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing aspects and many of the attendant advantages of this invention will become more readily appreciated as the same become better understood by reference to

the following detailed description, when taken in conjunction with the accompanying drawings, wherein:

FIGURE 1 shows a graph comparing the number of clones (colony forming units, CFU) obtained after transformation as a function of cell density (OD600) at the time of harvesting cells, as described in EXAMPLE 1. Transformation frequency of E. coli with supercoiled control DNA is shown as "Δ– -", and transformation frequency with vector and PCR product containing the attB sequences leading in vivo recombination is shown as "—♦—". The right y-axis represents the number of clones obtained with the control DNA, the left y-axis represents the number of clones obtained using the PCR product.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Unless specifically defined herein, all terms used herein have the same meaning as they would to one of ordinary skill in the art.

In one aspect, the invention provides methods for cloning polymerase chain reaction (PCR) products by site-specific recombination *in vivo*. Site-specific recombinases are proteins that are present in many organisms (*e.g.*, viruses and bacteria) and have been characterized to have both endonuclease and ligase properties. These recombinases (along with associated proteins in some cases) recognize specific sequences of bases in DNA and exchange the DNA segments flanking those segments. The recombinases and associated proteins are collectively referred to as "recombination proteins" or "recombination proteins and accessory proteins" (*see, e.g.*, Landy, A. (1993) *Curr. Op. Biotechnol.* 3:699-707).

Numerous site-specific recombination systems from various organisms have been described (*see, e.g.*, Hoess et al. (1986) *Nucleic Acids Research* 14(6):2287; Abremski et al. (1986) *J. Biol. Chem.* 261(1):391; Campbell (1992) *J. Bacteriol.* 174(23):7495; Qian et al. (1992) *J. Biol. Chem.* 267(11):7794; Araki et al. (1992) *J. Mol. Biol. 225*(1):25; Maeser & Kahnmann (1991) *Mol. Gen. Genet.* 230:170-176; Esposito et al. (1997) *Nucl. Acids Res.* 25(18):3605). Many of these belong to the integrase family of recombinases (Argos et al. (1986) *EMBO J.* 5:433-440). Perhaps the best studied of these are the Integrase/att system from bacteriophage lambda (Landy (1993) *Curr. Op. Genet. Devel.* 3:699-707), the Cre/loxP system from bacteriophage P1 (Hoess &

Abremski (1990) In *Nucleic Acids Mol. Biol.* 4:90-109; Eckstein & Lilley, eds, Berlin-Heidelberg, Springer-Verlag), and the Flp/FRT system from the *Saccharomyces cerevisiae* 2μ circle plasmid (Broach et al. (1982) *Cell* 29:227-234).

The major advantage of using integrases is that, unlike cut and paste technology with restriction enzymes and DNA ligase, integrases catalyze a concerted recombination. The very long sequence-specificity of the integrase reaction allows them to catalyze precise manipulations with large DNA molecules that would be difficult with restriction enzyme techniques.

The prototypical site-specific recombination reaction is used to integrate the lysogenic bacteriophage, lambda, into the *E. coli* chromosome. Two DNA sequences are involved, *attP*, the 243 bp phage attachment sequence, and *attB*, the 25 bp bacterial site of attachment (Mizuuchi & Mizuuchi (1980) *Proc. Natl. Acad. Sci. U.S.A.* 77:3220-4; Mizuuchi & Mizuuchi (1985) *Nucleic Acids Res.* 13:1193-208). Recombination catalyzed by the integrase protein, Int, occurs within a 15 bp "core" region of identity between *attP* and *attB*. Recognition of these sequences by integrase primarily involves the "arm" sequences of *attP*, which extend about 150 bases to the left and 100 bases to the right of the core (Mizuuchi & Mizuuchi (1980) *Proc. Natl. Acad. Sci. U.S.A.* 77:3220-4). If the core sequence in *attP* is replaced with another sequence, and there is an identical sequence on another DNA molecule that can pair with the new core, recombination can also take place. Integration causes the phage chromosome to be covalently attached to the bacterial chromosome, flanked by hybrid sequences called *attL* and *attR*. This is the BP reaction, because it involves *attB* and *attP* as substrates. The host integration host factor (IHF) is also required (Miller et al. (1980) *Cell* 20:721-9). The BP reaction requires the *attP* DNA to be supercoiled but *attB* DNA can be linear, like a PCR product.

Reversal of the reaction, used to excise lambda from the chromosome, additionally involves the phage protein Xis and restores the original *attB* and *attP* sequences. This LR reaction does not require supercoiled DNA and, in fact, works much better when the DNA is relaxed or linear. The BP and LR reactions are well characterized, function at room temperature, involve relatively stable proteins, and are robust.

Another site-specific recombination system is the yeast Flp recombinase, which is used in a recombination coupled to replication of the yeast 2μ plasmid (Sadowski (1995) *Prog. Nucleic Acid Res. Mol. Biol.* 51:53-91). Unlike integrase, the yeast Flp recombinase functions primarily in one direction, catalyzing recombination between two DNA sequences (FRT) that are antiparallel in the plasmid and thereby inverting the sequences between them. In the most common biotechnological applications of Flp/FRT, the FRT sequences are set up in parallel orientation and therefore the reaction leads to excision of the DNA between them (Hoang et al. (1998) *Gene* 212:77-86; Theodosiou et al. (1998) *Methods* 14:355-65). Accordingly, some embodiments of the methods and the vectors of the invention use the lambda or the Flp site-specific recombination systems. The use of other site-specific recombination systems is also within the spirit and scope of the invention.

In one aspect, the invention provides methods for cloning PCR products without the need for *in vitro* provision of restriction enzymes, ligation enzymes, or DNA purification steps. According to these methods, a PCR product is cloned *in vivo* using a site-specific recombination system. In some embodiments, the methods include the steps of (1) providing a PCR product flanked by a first site-specific recombination site and a second site-specific recombination site; and (2) transferring the PCR product into a cell comprising a target sequence flanked by a first recombination site partner and a second recombination site partner, and at least one recombination protein that mediates recombination between the first site-specific recombination site and the first recombination site partner, and between the second site-specific recombination site and the second recombination site partner.

The PCR product used in the methods of the invention typically is linear. Linear DNA has been previously used to transform bacterial cells, such as *Bacillus subtilis* (Contente & Dubnau (1979) *Plasmid* 2(4):555-71), *Lactobacillus plantarum* (Thompson et al. (1997) *Lett. Appl. Microbiol.* 35(6):419-25), and *E. coli* (Hoekstra et al. (1980) *J. Bacteriol.* 143(2):1031-2). However, these methods have involved either transforming DNA possessing an origin of replication and selecting for recircularization of the plasmid DNA (Conley & Saunders (1984) *Mol. Gen. Genet.* 194(1-1):211-8; Conley (1986) *Nucl. Acids Res.* 14(22):8919-32), or transforming DNA containing regions of homology with the host organism's genome, and selecting for integration of the DNA into the

chromosome or a plasmid containing homologous DNA (Dabert & Smith (1997) *Genetics* 145(4):877-89; Yu et al. (2000) *Proc. Natl. Acad. Sci. U.S.A.* 97(11):5978-83; Lee et al. (2001) *Genomics* 73(1):56-65). In contrast, the methods of the invention use site-specific recombination.

In the methods of the invention, at least three elements are brought together in a cell to clone a PCR product into a target sequence: (1) a PCR product flanked by a first and second recombination sites; (2) a target sequence flanked by a first and second recombination site partners; and (3) one or more recombination proteins to mediate recombination between the recombination sites and the recombination site partners. Moreover, the genetic background of the recipient cell should be conducive to recombination. For example, the use of mutant cells that are missing a protein that might interfere with recombination, such as *recD*, may be desirable.

The PCR product used in accordance with the invention typically includes two site-specific recombination sites which allow the PCR product to be transferred into one or more target sequences. The PCR product is typically generated using a set of primers to provide the first and second recombination sites to the PCR product. The PCR product may be any nucleic acid molecule derived from any source and may include non-naturally occurring nucleic acids. Additionally, the PCR product may comprise a particular sequence of interest (*e.g.*, a gene) or it may comprise a population of molecules (*e.g.*, molecules generated from genomic or cDNA libraries).

In some embodiments, libraries (*e.g.*, populations of genomic DNA or cDNA, or populations of nucleic acid molecules, produced by *de novo* synthesis such as random sequences or degenerate oligonucleotides) are utilized in accordance with the present invention. By the recombination methods of the invention, the library may be easily inserted into different target sequences. These target sequences may be genomic sequences or they may be on plasmid vectors (or combinations of vectors). Such vectors may be introduced into different host systems (prokaryotic and eukaryotic) to evaluate and analyze the library or a particular sequences or clones derived from the library. Alternatively, vectors containing the cloned PCR products may be used *in vitro* systems such as *in vitro* expression systems for production of RNA and/or protein.

The target sequence for site-specific recombination of the PCR product according to the methods of the invention may be a genomic sequence, or it may be a plasmid

sequence. The methods of the invention can be used with any genomic target sequences, prokaryotic or eukaryotic. Similarly, the methods may be used with target sequences on plasmid vectors that may function in a variety of systems or host cells, for example, prokaryotic vectors, eukaryotic vectors or vectors which may shuttle between various prokaryotic and/or eukaryotic systems (*e.g.*, shuttle vectors). Prokaryotic plasmid vectors for use in the invention include but are not limited to vectors which may propagate and/or replicate in gram negative and/or gram positive bacteria, including bacteria of the genus *Escherichia*, *Salmonella*, *Proteus*, *Clostridium*, *Klebsiella*, *Bacillus*, *Streptomyces*, and *Pseudomonas*, *Sinorhizobium*, and *E. coli*. Eukaryotic vectors for use in the invention include vectors which propagate and/or replicate and yeast cells, plant cells, mammalian cells (*e.g.*, human cells), fungal cells, insect cells, fish cells and the like. Particular plasmid vectors of interest include but are not limited to cloning vectors, sequencing vectors, expression vectors, fusion vectors, two-hybrid vectors, gene therapy vectors, and reverse two-hybrid vectors. Such vectors may be used in prokaryotic and/or eukaryotic systems depending on the particular vector.

Typically, the target sequence comprises a marker gene that permits identification of site-specific recombination events and that is flanked by two recombination site partners. The marker sequence may be a toxic gene such that only cells in which the toxic gene has been replaced by the PCR product are able to grow. Alternatively, the marker gene may produce a selectable phenotypic change in cells, such as is seen, for example, after expression of β-galactosidase. Any marker may be used that enables the selection for transfer of the PCR product into the target sequence, for example β-glucuronidase (GUS) or green fluorescent protein (GFP), or fusions between GUS and GFP. The marker gene expression may be constitutive, or it may be inducible.

According to the methods of the invention, no purification of PCR products in the PCR reaction is required before introducing them into a target sequence by site-specific recombination *in vivo*. Thus, aliquots from the PCR reaction may be directly transferred into a cell. Alternatively, PCR products can be purified if desired, for example, by PEG precipitation.

Typically, the PCR product is transferred into cells containing a source of one or more recombination proteins for cloning the PCR product into the target sequence. In some embodiments, a source of one or more recombination proteins is introduced into the

cell along with the PCR product. The source of one or more recombination proteins is typically transient. For example, the source of recombination protein(s) may be a plasmid that is removed from the cells after the site-specific recombination reaction has occurred. Alternatively, the expression of the recombination protein(s) may be transiently induced.

The PCR product may be introduced into the cell by a high frequency method, for example by electroporation. Other methods of transferring DNA into cells are within the scope of the invention. The PCR may be introduced into a cell already comprising the target sequence, for example in the genome or on a plasmid. Alternatively, the target sequence may be introduced into the cell at the same time as the PCR product.

In some embodiments, the methods of the invention use the integrase/att system from bacteriophage lambda to direct site-specific recombination. Accordingly, the starting point for cloning PCR products is a target sequence flanked by the two recombination site partners that are *attP* sites, *attP*1 and *attP*2. The two *attP* sites flank a marker gene that permits the identification site-specific recombinants. For example, the marker gene may be *ccdB*, a toxin gene that is lethal in *E. coli* strains lacking the antitoxin, *ccdA* or a mutated DNA gyrase (Bahassi et al. (1999) *J. Biol. Chem.* 274:10936-44; Bernard et al. (1993) *J. Mol. Biol. 234*:534-41). If *ccdB* is replaced by the PCR product by site-specific recombination, the target sequence is no longer toxic to *E. coli* strains lacking the antitoxin. Thus, only recombinants in which the toxic gene is replaced by the PCR product will permit the growth of *ccdA*-minus *E. coli*. Alternatively, the marker gene may be any other gene that provides a phenotype that can be selected for or against. As noted above, the target sequence may be a chromosomal sequence or it may be on a plasmid.

PCR products are synthesized using PCR primers that contain the *attB*1 and *attB*2 sites. For example, the PCR products may be cloned by first adding gene specific primers, carrying out the PCR reaction for several rounds, then adding the *attB* primers and running the PCR reaction to completion. Nested PCR may be performed, starting with primers containing only 12 bp of the *attB* sequence in the first rounds of synthesis then amplifying these using primers that contain the entire *attB* sequence (*see, e.g.*, http://www.invitrogen.com/). The second set of primers is standard and thus less expensive.

The PCR primers may include additional nucleotides at their ends. Lengthening the primers in this way may allow some degradation of the ends to occur without destroying the recombination sites needed for site-specific recombination. In addition, the PCR primers may contain *chi* sequences. *Chi* sequences are recognized in the functioning of certain recombination pathways in *E. coli*. For example, the *E. coli* *recBCD* nuclease is sequence-specific. Recognition of the *chi* sequences is orientation-dependent with respect to a double stranded gap that initiates *recBCD* binding. The presence of *chi* sequences, or similar sequences, on the PCR primers may enhance the efficiency of the methods of the invention (Dabert & Smith (1997) *Genetics* 145(4):877-89).

The PCR reaction may be carried out with a *Pfx* proofreading polymerase (Cline et al. (1996) *Nucleic Acids Res.* 24:3546-51) to keep the error rate low. To decrease the problem of incorrect priming, polymerase that is blocked by an antibody may be used (Kellogg et al. (1994) *Biotechniques* 16:1134-7). Thus, PCR reactions are set up at room temperature and when the temperature is raised, the antibody is denatured and the reaction begins. DNA in PCR reactions may be monitored using fluorescence. DMSO and glycerol may be used in the PCR reactions to reduce problems caused by DNA with a high G/C content (Moreau et al. (1997) *Methods Mol Biol.* 67:47-53; Varadaraj & Skinner (1994) *Gene* 140:1-5).

In some embodiments, the PCR product is transformed into *E. coli*. The *E. coli* strain typically has a mutation in the *recD* gene, which codes for one of the major endonucleases. It has been shown that mutating the *recD* gene stabilizes linear DNA. Therefore, the *recD* mutation may allow for better survival of the linear PCR product as it enters the cell. The *E. coli* strain may additionally have mutations in other genes, such as *recA*, *endA*, *sbcA*, *sbcB*, *recB*, or *recC*, in order to increase the efficiency of linear DNA uptake and survival. The presence of lambda *red* genes may also be desirable, as these genes enhance recombination in recombination deficient strains of *E. coli* (Datsenko & Wanner (2000) *Proc. Natl. Acad. Sci. U.S.A.* 97(12):6640-5).

The *E. coli* strain also contains a source of lambda integrase. Typically, the integrase gene may be provided on a plasmid under the control of an inducible promoter, for example, the *lac* promoter which is inducible by IPTG. The plasmid may additionally have a temperature-sensitive origin of replication such that the plasmid may be cured by

incubating the cells at high temperatures. Plasmids that express lambda integrase are available (*see, e.g.*, Platt et al. (2000) *Plasmid* 43:12-23).

The *E. coli* cells expressing the lambda integrase are made competent using a modification of standard techniques, as described, for example, in EXAMPLE 1. In some embodiments, the cells are harvested during the stationary phase of growth for competence induction. As cells enter stationary phase, the expression of Integration Host Factor (IHF) is induced. IHF is required for lambda integrase function and may be responsible for the transformation frequency increase under these conditions. Therefore, the efficiency of transfer of the PCR product into the vector may be increased by using cells made competent during stationary phase, as shown in FIGURE 1. Alternatively, another source of IHF or other enhancing factors may be provided. For example, the IHF coding sequences may be provided operably linked to an inducible promoter. This would permit the cells to be harvested during an earlier phase of growth, which is the peak competence stage (see FIGURE 1).

The PCR product may be transformed into *E. coli* simultaneously with the target sequence. The target sequence may be a genomic sequence, or it may be a plasmid sequence

Alternatively, the target sequence may be transformed into *E. coli* first, provided that a nontoxic marker gene is used. Without being bound to any particular theory of operation, it is likely that lambda integrase recognizes the *attB1* and *attP1* sites, and the *attB2* and *attP2* sites, and mediates recombination between the respective site pairs. The recombination replaces the marker gene, for example the toxic *ccdB* gene, with the PCR product and creates *attL1* and *attL2* sites flanking the PCR product sequence.

Using the methods of the invention, the cloning of a PCR product occurs entirely *in vivo*, without the need for restriction enzymes, ligation enzymes, or DNA purification steps. Once the PCR product is cloned into the vector, it can then be moved into any number of other genomic sites or plasmid vectors using site-specific recombination (*see, e.g.*, Hartley et al. (2000) Genome Res. 10:1788-95; Walhout et al. (2000) *Science* 287:116-22; Walhout et al. (2000) *Methods Enzymol.* 328:575-92). Therefore, the methods of the invention are faster, easier, less expensive, and provide a larger variety of uses than traditional PCR cloning procedures.

In some embodiments, the methods of the invention provide a frequency of cloning PCR products into a target sequence *in vivo* of almost 100%. Thus, almost 100% of selected cells obtained using the methods of the invention contain target sequences in which the marker gene has been replaced by the PCR product. In some embodiments, the methods of the invention provide a frequency of cloning PCR products into a target sequence *in vivo* of about 80%. In some embodiments, the methods of the invention provide a frequency of cloning PCR products into a target sequence between about 50% and about 95%.

In some embodiments of the invention, the methods for cloning PCR products use a site-specific recombination system other than the integrase/att system from bacteriophage lambda. Other methods may employ other bacteriophage recombination systems (*e.g.*, $\phi$80, P2, P4 or Cre/*loxP* of P1), or other non- bacteriophage recombination systems (*e.g.*, Flp/FRT of yeast). In some embodiments, the site-specific recombination event occurs in cells other than *E. coli*.

In another aspect, the invention provides site-specific recombination kits for cloning PCR products by site-specific recombination *in vivo*. In some embodiments, the kits of the invention comprise competent cells of a genotype able to carry out the recombination reaction (*e.g.*, V355(pBH-INT)), a suitable vector plasmid (*e.g.*, pMK2010), a control PCR product or primers sufficient for generating such PCR product. The kits may also include a set of secondary primers (*attB* primers) for a nested PCR protocol. These primers may contain modified DNA bases or intrabase linkages.

EXAMPLE 1

This Example describes a representative method of the invention using the integrase/att system from bacteriophage lambda as the site-specific recombination system.

PCR products were generated for the *lacZa* gene of *E. coli* using primers that cause the product to be flanked by bacteriophage lambda *attB1* and *attB2* sequences. Two sets of primers were used (primary and secondary) in a nested PCR protocol. The primary primer sequences were 5' GGA GGC TCT TCA ATG ACC ATG ATT ACG GAT TC 3' (SEQ ID NO:1) and 5' AGC TGG GTT CTA CGC CGA GTT AAC GCC ATC AA 3' (SEQ ID NO:2). The secondary sequences were 5' GGG GAC AAG TTT

GTA CAA AAA AGC AGG CTT AGG AGG CTC TTC AAT G 3' (SEQ ID NO:3) and 5' GGG GAC CAC TTT GTA CAA GAA AGC TGG GTU CTA 3' (SEQ ID NO:4). The PCR product and the plasmid vector pMK2010 were co-electroporated into electrocompetent cells of *E. coli* strain V355 (pBH-INT). V355 (CGSC6720) is a *recD* mutant, inactivating one of the major exonucleases of *E. coli*. Plasmid vector pMK2010 contains the *ccdB* gene, which is toxic to most *E. coli strains*, flanked by *attP1* and *attP2* sites. pBH-INT contains the gene encoding lambda integrase under control of the lactose operon repressor (*i.e.*, inducible by IPTG), and a temperature-sensitive origin of replication.

V355 (pBH-INT) electrocompetent cells were prepared by growing an overnight culture in LB Broth containing 100 μg/ml ampicillin, diluting the culture 1:100 into LB Broth containing 100 μg/ml ampicillin and 20 μg/ml IPTG, and growing the culture by shaking at 30°C and 240 r.p.m. until the $OD_{600}$ reached 2.4 to 2.6 (about 4 to 6 hours). The cells were prepared for electroporation by successive washes in 10% glycerol, and the final cell pellet were resuspended in 1/250 of the original culture volume of 10% glycerol. At this point, the cells were distributed into 45 to 90 μl aliquots, frozen with liquid nitrogen, and stored at -80°C.

3 μl of the PCR product (3000 ng/microliter) and 1 μl of pMK2010 (150 ng/microliter) were co-electroporated into a 50 μl aliquot of competent cells. The cells were grown out in SOC medium at room temperature, which is the optimal temperature for lambda integrase function, then plated on onto LB Broth plates containing 75 μg/ml kanamycin to select for the pMK2010 derivative, and incubated at 42°C overnight to cure the cells of the pBH-INT plasmid. Between 400-600 recombinant clones were obtained per electroporation. Based on restriction enzyme analyses, 23 out of 24 clones contained correctly cloned PCR products.

Cells grown to different culture densities were prepared for electroporation and transformed either with control plasmid to test cell competence for transformation with supercoiled DNA or transformation with vector plasmid and the PCR product containing the *attB* sequences. Even though peak electrocompetence occurs when cells are harvested during late logarithmic growth phase, peak numbers of cloned PCR products were obtained when cells were well into stationary phase, at an $OD_{600}$ of about 2.5, as shown in FIGURE 1. The peak represents ~300 cloned PCR products per

electroporation. The data show that successful transformation leading to *in vivo* recombination requires a different physiological state of the cell than does transformation with supercoiled DNA, with maximum competence for recombination occurring much later in cell growth. This may be due to the presence of integration host factor (IHF), which is required for integrase function, in stationary phase cells. Consistent with this, expression of IHF in early growth phase produces a better efficiency of transformation with cloned PCR products in early growth phase and peak numbers of cloned PCR products are obtained earlier in the growth phase.

While the preferred embodiment of the invention has been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.